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(54) Title: **SHARK CARTILAGE EXTRACS AND USB THEREOF FOR IMMUNOMODULATION**

姜明

(57) Abstract: Disclosed is a composition from shark cartilage comprising immunoactive proteoglycans and other immunoactive components having a molecular weight greater than about 100 KD, wherein the composition has a uronic acid content of about 0.05-0.5 µg glucuronic acid per µg of the composition. The composition may be useful for treating or preventing tumor growth, bacterial infections, viral infections and/or fungal infections.

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SHARK CARTILAGE EXTRACS AND USE THEREOF FOR IMMUNOMODULATION

Field of the Invention

The present invention relates to compositions comprising active principles from shark cartilage, methods
5 of producing such compositions and use of such compositions as immunomodulators.

Background of the Invention

Shark cartilage has traditionally been credited with a number of medical benefits but it is primarily
10 marketed for its anticancer effects [1-5]. The potential value of shark cartilage in cancer treatment as an angiogenesis inhibitor [6-8] has been investigated and several clinical trials have been done or are underway. However, its efficacy has been controversial thus far [9-
15 14].

In addition, there have been a number of other bioactivities attributed to shark cartilage, including beneficial effects on osteoarthritis, rheumatoid arthritis, progressive systemic sclerosis and neurovascular glaucoma,
20 just to name a few [15-17]. There are suggestions that shark cartilage also contains anti-inflammatory agents and wound healing substances [15,18,19]. There has been speculation, rather than scientific evidence, that shark cartilage may stimulate the cellular and humoral components
25 of the immune system, presumably making it effective against tumor growth and bacterial, viral and fungal infections. Several shark cartilage preparations claiming immune-boosting effects are on the market (Cartilade™ from Cartilage Technologies Inc.; SC Formula, from Nature's
30 Sunshine, etc). An extract of shark liver has recently been found to stimulate the immune system [20] and Rosen et al

[21] reported on immunoregulatory effects of a bovine cartilage preparation (CatrixS™). They disclose that fractions with 0-10 KD and 30-100 KD range enhanced T-dependent and T-independent antibody responses *in vivo*, possibly related to the chondroitin sulfate component.

A variety of shark cartilage products have been introduced as dietary supplements or even tested in clinical practice, however, many of the claims of purity, bioactivity and clinical efficacy are not backed by rigorous scientific investigation. Although there has been progress in the understanding of the field, the isolation, purification, analysis and standardization of these materials are still extremely challenging.

There has been controversy as to whether shark cartilage could be effective via oral administration. The active principles of shark cartilage described in the literature and believed to be behind various activities are biopolymers, usually proteins. Pettit and Ode isolated two glycoproteins from shark cartilage: sphyrnastatin 1 and 2 [22]. McGuire et al demonstrated antiproliferative activity of a heat stable, <10 KD fraction of shark cartilage extract on endothelial cell population [4]. This is in agreement with Oikawa's work, where a substance with an average molecular mass of 1-10 KD was isolated from shark cartilage. Oikawa's material was heat stable, however no information on the composition of the substance was given and the material was not administered orally [23]. Suzuki et al isolated from shark cartilage a fraction of large molecular mass inhibiting solid tumor growth [24]. Another preparation, a patented anti-inflammatory substance isolated from shark cartilage, was of molecular mass larger than 100 KD [25], however this substance was not intended for oral administration.

Proteins have low oral bioavailability and short *in vivo* half-lives, which to date have necessitated their delivery by infusion or frequent injections [26]. In the case of antigen delivery, biodegradable and biocompatible microspheres have been used for antigen delivery into the GI tract [27]. Conventional wisdom says that biopolymers would be either disintegrated into monomers, or smaller or larger oligomers, and thus lose biological activity, (a classical problem with protein or peptide based pharmaceuticals), or if they remained unchanged or were cleaved into large non-absorbable fragments, they would be incapable of exerting their biological effect due to physical barrier.

Except for the reports from questionable clinical trials and other anecdotal evidence [28,29,30] there are only a limited number of published scientific papers demonstrating biological effects due to oral administration of shark cartilage preparations. As an example, one study demonstrated a positive effect of shark cartilage on the survival time of rats with implanted intracranial tumor [31].

The protein and carbohydrate components of shark cartilage extracts come largely from proteoglycans (PG) and represent up to 50% of its dry weight [32]. Cartilage also contains collagen and glycosaminoglycans (GAGs), including chondroitins A, B, and C. The GAG part of PG is known to inhibit proliferation of fibroblasts. A number of GAGs, such as heparin and heparan sulfate, have been shown to interact with a wide range of growth factors and cytokines, including α -FGF, β -FGF, GM-CSF, IL-1a, IL1b, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, hIL-10, vIL-10 and INF- γ . This interaction has been shown to be important in modulating the activity of these growth factors. Shark cartilage contains

PGs, GAGs (e.g. CHS A, 1 and C) and collagen. PGs stimulate the immune system, which works synergistically with protein to fight disease [33].

Proteoglycans might be involved in the interaction of primitive, hematopoietic progenitor cells and stromal cells. IL-3 and GM-CSF can be bound by heparan sulfate. PGs form bone-marrow stromal cells or their extracellular matrix and can be presented in a biologically active form of hematopoietic cells. Proteoglycans might also be involved in cell adhesion and homing of hematopoietic stem and progenitor cells [34].

Summary of the Invention

There is provided a composition from shark cartilage comprising proteoglycans and an immunoactive component having a molecular weight greater than about 100 KD, wherein the composition has a uronic acid content of about 0.05-0.5 μ g glucuronic acid per μ g of the composition.

There is further provided a formulation comprising an immunomodulating effective amount of a composition from shark cartilage containing proteoglycan and an immunoactive component having a molecular weight greater than about 100 KD, together with a physiologically acceptable carrier, excipient and/or diluent, wherein the composition has a uronic acid content of about 0.05-0.5 μ g glucuronic acid per μ g of the composition.

There is yet further provided a use of an immunomodulating effective amount of a composition from shark cartilage containing proteoglycans and an immunoactive component having a molecular weight greater than about 100 KD, wherein the composition has a uronic acid content of

about 0.05-0.5 μg glucuronic acid per μg of the composition, for modulating an immune system of a subject in need of immunomodulation.

Still further, there is provided a method
5 comprising administering to a subject in need of immunomodulation an immunomodulating effective amount of a composition from shark cartilage containing proteoglycan and an immunoactive component having a molecular weight greater than about 100 KD, wherein the composition has a uronic acid
10 content of about 0.05-0.5 μg glucuronic acid per μg of the composition.

Still yet further, there is provided a commercial package comprising an immunomodulating effective amount of a composition from shark cartilage containing proteoglycan and
15 an immunoactive component having a molecular weight greater than about 100 KD, wherein the composition has a uronic acid content of about 0.05-0.5 μg glucuronic acid per μg of the composition, together with instructions for its use in modulating an immune system of a subject in need of
20 immunomodulation.

Detailed Description

Abbreviations:

α -FGF	acidic fibroblast growth factor
APMSF	4-amidinophenylmethanesulfonyl fluoride HCl
β -FGF	basic fibroblast growth factor
BCA	bicinchoninic acid
BSA	bovine serum albumin
CHS	Chondroitin sulfate
DMAB	p-dimethylamino benzaldehyde
DMB	1,9-dimethyl-methylene blue
ELISA	enzyme linked immunosorbent assay
GAG	glycosaminoglycan
GM-CSF	granulocyte-macrophage colony stimulating factor
GuCl	Guanidine hydrochloride
IL	interleukin
INF- γ	interferon gamma
KD	kilodalton
LPS	lipopolysaccharide
mAb	Monoclonal antibody
MD	megadalton
mw	Molecular weight
MWCO	molecular weight cut-off
MOPS	3-(N-morpholino)-propane sulfonic acid
Na ₂ EDTA	ethylene tetraacetic acid disodium salt
NK	natural killer
PG	proteoglycan
PBS	phosphate buffered saline
PMSF	Phenylmethanesulfonyl fluoride
PP	Peyer's patches
SEC	Size exclusion chromatography
TBS	tris buffered saline
TMB	tetramethyl benzidine substrate
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone

Compositions:

Shark cartilage compositions of the present invention comprise an immunoactive component having a molecular weight greater than about 100 KD. The average molecular weight is about 500 KD. In particular, components having a molecular weight greater than about 500 KD, even more particularly greater than about 1 MD, are implicated in the immunoactivity exhibited by the compositions.

One category of components that make up the shark cartilage immune boosting preparation are proteoglycans. Proteoglycans are a class of glycosylated proteins, which have covalently linked sulfated glycosaminoglycans, (i.e., chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, keratan sulfate). Glycosaminoglycans are sulfated polysaccharides made of repeating disaccharides (typically a repeat of 40-100 times), which consist of uronic acid (or galactose) and hexosamines. Components of the disaccharides are (glucuronic acid/iduronic acid -N-acetylgalactosamine) in chondroitin sulfate and dermatan sulfate, (glucuronic acid/iduronic acid -N-acetylglucosamine) in heparan sulfate and heparin, and (galactose -N-acetylglucosamine) in keratan sulfate.

Uronic acid is a component of proteoglycans. In shark cartilage compositions of the present invention, the uronic acid content is preferably from about 0.05 to about 0.5 μg glucuronic acid per μg of the composition, more particularly from about 0.08 to about 0.3 $\mu\text{g}/\mu\text{g}$.

Proteoglycan content of shark cartilage compositions of the present invention may be measured as the index of proteoglycan content, which is defined as the ratio of the composition's proteoglycan content to the

proteoglycan content of the supernatant of 40% ammonium sulfate precipitation. The index of proteoglycan content is preferably from about 0.1 to about 1.0, more particularly from about 0.3 to about 0.8.

5 While it is believed that the immunoactive components of the composition are proteoglycans, the immunoactive components may be different from proteoglycans or may be a combination of proteoglycans and other component of the composition.

10 Proteins are present in the composition in an amount of about 20-30% by weight of the composition, particularly about 25% by weight. All major amino acids were present. Based on a weight percentage of total amino acid content, glycine content may be from about 14-16%,
15 proline content may be from about 14-15%, alanine content may be from about 12-14%, glutamic acid content may be from about 10-12%, aspartic acid content may be from about 6-7% and arginine content may be from about 5-7%. The predominance of glycine and proline suggests the presence of
20 significant amounts of collagen in the compositions.

本頁 17 30.8%

Methods of preparation:

Shark cartilage compositions of the present invention are typically prepared from shark cartilage by extraction methods. Generally, compositions are prepared by
25 extracting the active immunomodulating principles from shark cartilage into an extraction medium, separating unwanted solid materials from the extract, and lyophilizing or freeze-drying the extract to yield solid shark cartilage composition.

30 There are a number of additional steps that may be performed in a variety of combinations in connection with

the generalized method. The shark cartilage may be initially ground or pulverized to increase efficiency of extraction. Unwanted solids may be washed and the washings further combined with the extract to increase yield of the active immunomodulating principles. The extract may be reduced in volume before lyophilization or freeze-drying. The extract may be dialyzed and/or fractionated by ultrafiltration before lyophilization or freeze-drying. The active immunomodulating principles may be precipitated from the extracts and redissolved in another medium before lyophilization. Chromatography may be performed on the lyophilized composition.

Grinding or pulverizing shark cartilage may be performed by any suitable method known in the art. For small samples, a mortar and pestle is appropriate. For large samples, grinding mills or industrial scale pulverizers are more useful. The extent of grinding or pulverizing is not important.

A variety of extraction media may be used. Aqueous media, with or without additional extraction aids, are preferred. Extraction may be typically performed at temperatures from about 0°C-100°C. Temperatures in excess of 100°C may be used, but extractions at these temperatures are typically done under pressure.

When water is used alone, it is preferred to perform the extraction at an elevated temperature, typically at a temperature of from about 55°C-95°C, more preferably from about 75°C-90°C. Extractions into hot water are typically done for about 0.5-3 hours, more particularly for about 1-2 hours. When lower temperatures (ambient, 4°C, etc.) and longer times are used for the extraction,

preservatives, such as NaN_3 , can be used to prevent bacterial growth.

A number of chaotropic agents may be added to the extraction medium to facilitate extraction. Monovalent

5 salts such as halides with monovalent or divalent cations (e.g. LiCl , CaCl_2 , MgCl_2 , LaCl_3) and guanidine (e.g. guanidine hydrochloride (GuCl)) are preferred, with GuCl being a particularly preferred chaotropic agent. The chaotropic agent is preferably used in an amount of up to
10 the saturation point, preferably from about 2-6 M. Extractions with aqueous media comprising chaotropic agents are typically done at lower temperatures but for longer times than extractions with pure water. Typically the extraction temperature is from about 0°C - 25°C , more
15 particularly from about 2°C - 10°C . Extraction time is typically on the order of about 12-96 hours, more particularly from about 24-72 hours.

Other additives may be present in the extraction medium. The extraction medium may be buffered using any
20 number of a great variety of buffers known in the art (citrate, tris, MES, ADA, PIPES, MOPS, etc.). A preferred buffer is MOPS, providing a buffered pH of about 6.5. Complexing agents (such as EDTA, citrate, hydroxypyridones, etc.), protease inhibitors (such as benzamidine,
25 1,10-phenanthroline, TPCK, PMSF, APMSF, etc.), and other additives may also be used.

Typically about 2-50 volumes, particularly about 2-5 volumes of the extraction medium is used for the extraction of the active immunomodulating principles. In
30 addition, the extraction is typically performed under gentle agitation, which may be achieved by agitating the extraction

vessel, by mechanically stirring the slurry or through the action of heating the slurry.

Once the extraction period is complete, the extract containing the active immunomodulating principles is typically separated from any unwanted solid material. Separation may be accomplished by any method known in the art, such as centrifugation or filtration. Cross-flow filtration and vibrating membrane filtration followed by microfiltration are preferred methods of filtration. However, centrifugation is preferred over filtration, although microfiltration can be used in conjunction with centrifugation. Centrifugation at 150-25,000 g for a time of about 15-60 minutes is generally satisfactory. One skilled in the art will understand that centrifuge speed and time are related and will be able to adjust speed and/or time accordingly to achieve the desired separation.

The extract may be used in subsequent steps or the unwanted solids may be washed and the washings combined with the extract. Washing may be done with adequately preserved water or with fresh extract and may be done any number of times. The extract, with or without added washings, may be reduced in volume by evaporation if desired.

The extract may be subject to direct lyophilization or freeze-drying to obtain a shark cartilage composition comprising the active immunomodulating principles. Lyophilization or preferably spray-drying are used in the drying step.

The extract may be subjected to other purification steps before or after lyophilization or freeze-drying. Dialysis is typically performed before the lyophilization/freezing-drying step. Dialysis may be done against any number of different aqueous media including, but

not limited to, diluted alcohols, various buffers (e.g. Tris buffer), diluted acetic acid and pure water, and may be performed against a membrane having a desired molecular weight cut off. Instead of or in addition to dialysis, the extract may be ultrafiltered through membranes by methods generally known in the art. A single ultrafiltration membrane or a series of membranes having increasing molecular weight cut-offs may be used. The molecular weight cut-off for such membranes is typically in the range of about 10-3000 KD, for example, 30 KD, 100 KD, 500 KD and 1000 KD. Dialysis could also be replaced by desalting with gel filtration media such as Sephadex™ G-25, Bio Gel™ P-6 or equivalents.

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分析方法

The active immunomodulating principles may be precipitated from the separated extract using a precipitant, such as ethanol or ammonium sulfate, instead of or in addition to the other purification steps. A final ethanol concentration of about 70-90% by volume, more preferably about 80%, is typical, while a final concentration of about 15-45% w/v, preferably 40%, is typical for ammonium sulfate. Such precipitation is typically carried out over a period of 12-24 hours in a refrigerator (e.g. about 2-10°C). The precipitated immunomodulating principle may then be redissolved in water or buffer and subsequent purification performed.

Subsequent to lyophilization, further purification may be accomplished by way of chromatography or ultrafiltration. Chromatographic fractionation may be achieved using size exclusion or ion exchange matrices such as Sephadex™, Sephacryl™ or DEAE Sepharose™ media. One skilled in the art will understand how to choose appropriate fractionation ranges, for example, Sephadex™ G-100 may be

used for a molecular weight cut off of about 100 KD.

Sephadex™ G-100, Sephacryl™ S-300 HR and DEAE Sepharose™

are preferred examples of chromatographic media. One

skilled in the art will be able to choose appropriate

5 eluants depending on the chromatographic matrix used and the

desired fractionation. Such eluents are well known in the

art and include, for example, buffers (such as acetate,

phosphate and Tris buffers) and aqueous sodium formate.

Acetate buffer is particularly preferred for DEAE Sepharose™

10 while aqueous sodium formate is particularly preferred for

Sephadex™ and Sephacryl™. Other additives may be used to

alter the solubility conditions and may be optimized for the

particular chromatographic matrix and/or eluant. For

example, sodium chloride and ethanol may be used in the

15 eluant systems.

Uses:

Compositions from shark cartilage produced by methods of the present invention are useful for stimulating the cellular and humoral components of the immune system.

20 Thus, the compositions may be useful for treating or

preventing tumor growth, bacterial infections, viral

infections and/or fungal infections. Specifically, the

compositions have significant stimulatory activity for mouse

spleen B cells and NK cells, as well as for mouse peritoneal

25 macrophages, when added to these cells in tissue culture.

Macrophages are the first link in the immune response chain

since they scavenge antigens, process them, and present the

peptides from those antigens thereby activating the immune

response cascade; B cells produce antibodies, and NK cells

30 are vital in protection from tumors.

The shark cartilage compositions of the present invention may be used as such or they may be formulated into

formulations. Formulations may be for enteral (e.g. oral), rectal, parenteral or other modes of administration. The formulations comprise the composition of the present invention in combination with one or more physiologically acceptable ingredients, such as carriers, excipients and/or diluents. Compositions and formulations for oral administration are particularly preferred.

Formulations may be prepared, for example, in unit dose forms, such as tablets, capsules, dragees, suppositories or ampoules. They may be prepared in a conventional manner, for example by means of conventional mixing, granulating, confectioning, dissolving or lyophilising processes.

To prepare formulations of the present invention in the form of dosage units for oral administration, the shark cartilage compositions of the present invention may take the form of, for example, granules, tablets, capsules, liquids or dragees prepared together with physiologically acceptable carriers, excipients and/or diluents.

Typical physiologically acceptable ingredients include:

- (a) binding agents such as starch (e.g. pregelatinised maize starch, wheat starch paste, rice starch paste, potato starch paste), polyvinylpyrrolidone, hydroxypropyl methylcellulose, gum tragacanth and/or gelatin;
- (b) fillers such as sugars (e.g. lactose, saccharose, mannitol, sorbitol), amylopectin, cellulose preparations (e.g. microcrystalline cellulose), calcium phosphates (e.g. tricalcium phosphate, calcium hydrogen phosphatelactose) and/or titanium dioxide;

(c) lubricants such as stearic acid, calcium stearate, magnesium stearate, talc, silica, silicic acid, polyethylene glycol and/or waxes;

(d) disintegrants such as the above-mentioned
5 starches, carboxymethyl starch, cross-linked polyvinylpyrrolidone, agar, alginic acid or a salt thereof (e.g. sodium alginate) and/or sodium starch glycollate;

(e) wetting agents such as sodium lauryl sulphate;
and/or,

10 (f) stabilizers.

Soft gelatin capsules may be prepared with capsules containing a mixture of the shark cartilage composition together with paraffin oil, liquid polyethylene glycols, vegetable oil, fat and/or another suitable vehicle
15 for soft gelatin capsules. Plasticizers such as glycerol or sorbitol may also be used. Hard gelatin capsules may contain granules of the composition. Hard gelatin capsules may also contain the composition in combination with solid powdered ingredients such as those listed above.

20 Liquid formulations for oral administration may be prepared in the form of solutions, syrups or suspensions. Liquid formulations typically comprise the shark cartilage composition together with an excipient such as sugar or sugar alcohols, and a carrier such as ethanol, water,
25 glycerol, propylene glycol, polyethylene glycol, almond oil, oily esters or mixtures thereof. If desired, such liquid formulations may also contain coloring agents, flavoring agents, saccharine, thickening agents (e.g. carboxymethyl cellulose), suspending agents (e.g. sorbitol syrup, methyl
30 cellulose, hydrogenated edible fats), emulsifying agents (e.g. lecithin, acacia), and/or preservatives (e.g. methyl

p-hydroxybenzoates, propyl p-hydroxybenzoates, sorbic acid). Liquid formulations for oral administration may also be prepared in the form of a dry powder to be reconstituted with water or another suitable vehicle prior to use.

5 An immunomodulating effective amount of the shark cartilage composition may depend on various factors, such as the method of administration, species of animal, age and/or individual condition. The composition may be administered in a range of about 50-5000 µg/day. In a normal case, the
10 approximate estimated daily dose for a human patient weighing approximately 75 kg is about 200-400 mg. The composition may be administered once per day or multiple times per day.

Brief Description of the Drawings

15 The invention will now be described by way of example having regard to the appended drawings in which:

 Figure 1A is a graph showing yields of fractions from ultrafiltration of (concentrated) shark cartilage extract 3. In Series a, 2 M guanidine hydrochloride was
20 used as the eluent; 64 ml of the solution containing 2.8 g was loaded to the ultrafiltration cell.

 Figure 1B is a graph showing immunoactivities in vitro of molecular weight fractions from ultrafiltration (guanidine hydrochloride) of concentrated extract 3 as
25 measured by thymidine incorporation ^{和. 和. 和}. Three different concentrations of sample are shown: 31.25 µg/ml: □; 62.5 µg/ml: ▢; 125 µg/ml: ▣. Controls were untreated cells (negative control) and cells challenged with 20 µg/ml LPS (positive control); they are denoted by □. Samples shown
30 are 3: undialyzed and lyophilized; 9a: MW<30 KD; 10a: 30-100

KD; 11a: 100-500 KD; 12a: 500-1000 KD; 13a: MW>1000KD. 9-13 are all dialyzed against a MWCO 12 KD membrane in water and lyophilized.

Figure 2 shows Sephadex™ G-100 size exclusion chromatography of extract 4. Fractions were monitored at 280 nm (Δ), and at 490 nm after mixing with phenol-sulfuric acid reagent (\diamond). One gram of sample was eluted with 1 M sodium formate, pH 3.5, at a flow rate of 24 ml/h, through a column of dimensions 2.6 x 100 cm.

Figure 3 shows DEAE Sepharose™ - Fast Flow ion exchange chromatography of extract 4. Initial eluent was 0.1 M sodium acetate, pH 4.4 with 0.15 M sodium chloride and 10% ethanol. Then the sodium chloride concentration was increased in a gradient up to 3 M. Flow rate was 4 ml/h through a column of dimensions 1 x 20 cm.

Figure 4 is spectrometric scans of DMB (16 μ g/ml) before (-) and after (-) complexation with extract 4 (100 μ g/ml, from 200-700 nm).

Figure 5 is an example of a titration curve generated as a relationship between the concentration of tested substance and immunoactivity *in vitro*. The immunoactivity *in vitro* was measured by thymidine incorporation.

Figure 6 is a graph showing proliferation of subpopulations of spleen cells (B cells and/or T cells) in the presence of 4. LPS was used as a B-cell stimulator whereas ConA was used as a T cell stimulator. Mixed spleen cells: \square ; purified B cells: \oplus ; purified T cells: \boxtimes .

Figure 7 is a graph showing stimulation of macrophages challenged with 4, as measured by production of

nitric oxide. The sample was tested at a concentration of 125 µg/ml (□) and 500 µg/ml (■). Cells alone were used as a negative control.

Figure 8 is a graph showing stimulation of macrophages by shark cartilage extract, determined by release of cytokines IL-6 (■) and IL-12 (□). Production of IL-6 and IL-12 was measured by sandwich ELISA.

Figure 9 is a graph showing a protective effect of feeding mice with 2, 4 or water from *Listeria*. Mice received 20 mg of 2 or 4 every second day for three weeks, then they received the lethal dose of pathogen and later sacrificed.

Examples

Materials and Methods:

All chemicals were of analytical grade and were purchased from Sigma Aldrich, (Milwaukee, WI), unless specified otherwise. The cartilage was obtained from healthy sharks (dog fish, *Squalus acanthias*) by freeing the cartilage of any adherent muscle or connective tissue and scraping it clean with scalpels and scissors. It was then stored at -80°C, until use. The cartilage was dried and pulverized to a mean particle size of 35 µm. The Amicon™ ultrafiltration cell (450 ml volume), and ultrafiltration membranes (76 mm in diameter) were obtained from Pall Corp (Mississauga, ON). Dialysis membranes (MWCO 12 KD) were obtained from Sigma Aldrich. All chromatography was performed using Pharmacia columns and matrices (Pharmacia, Uppsala, Sweden). RPMI 1640 medium was obtained from Gibco-BRL (Invitrogen Life Technologies, Burlington, ON). The BCA kit for protein determination was purchased from Pierce

(Rockford, IL). Costar™ 3950 ELISA and tissue culture plates were purchased from Fisher Scientific (Halifax, NS). Anti-Thy-1.2-coated and anti-B220 (anti-CD45R) Miltenyi™ microbeads and MidiMACS™ columns and magnets were supplied
5 by Miltenyi™ Biotek (Auburn, CA). All lyophilized extracts and fractions were obtained in the form of white, fluffy, flaky materials, unless specified otherwise.

Extraction:

Method A

10 Twenty grams of pulverized shark cartilage were suspended in 100 ml guanidine medium (4 M guanidine hydrochloride, 0.01 M Na₂EDTA, 0.1 M 6-aminohexanoic acid, 0.05 M sodium acetate, 0.001 M benzamidine hydrochloride, 0.01M N-ethylmaleimide, 0.001 M phenylmethylsulfonyl
15 fluoride and 0.02% sodium azide, pH 6.0), and gently stirred at 4°C for 24 hours. The solids were centrifuged (17,500 x g, 30 min), washed, and the combined supernatant was dialyzed against distilled water. After clearing by centrifugation the resulting material was lyophilized to
20 obtain 1; yield 1.1 g, 5.25%.

Method B

Twenty grams of pulverized shark cartilage were suspended in 100 ml of guanidine medium (2 M guanidine hydrochloride in 0.02 M MOPS, pH 6.5) and gently stirred at
25 4°C for 72 hours. The solids were centrifuged (13000 x g, 30 min.), washed, and the combined supernatant was dialyzed against 0.15 M NaCl in 0.05 M Tris buffer, pH 7.5. After clearing by centrifugation the resulting material was precipitated under gentle stirring with cold ethanol (final
30 ethanol concentration of 80%) and left in the fridge

overnight. The solids were filtered off, dissolved in the smallest volume of the above Tris buffer and dialyzed against distilled water. The sample was cleared by centrifugation and lyophilized to obtain 2; yield 0.61 g, 3.0%.

Method C

Three hundred grams of pulverized shark cartilage were suspended in 1500 ml of distilled water and extracted at 80-85°C for 1 hour. The solids were then removed by centrifugation (3000 x g, 30 min) and the pellet was washed twice with 400 ml of distilled water. The volume of the combined liquids was reduced by evaporation under vacuum to 500 ml and the concentrate was split into five equal portions that were treated as follows:

The first portion was directly lyophilized to obtain 3; yield 5.61 g, 9.4%.

The second portion was dialyzed against a 12 KD MWCO membrane and the dialyzed material was then lyophilized to obtain 4; yield 4.36 g, 7.3%.

The third portion was treated with ammonium sulfate (the final concentration of 40%) and gently stirred at 4°C for 16 hours. The solids were centrifuged off (18000 x g, 2 x 30 min.). The pellet was re-dissolved in 30 ml of distilled water, the material was dialyzed and finally freeze-dried to obtain 5; yield 2.63 g, 4.4%. The supernatant was also dialyzed and freeze-dried to obtain 6; yield 0.30 g, 0.5%.

The fourth portion was mixed with ethanol under stirring (the final ethanol concentration was 80%) and then gently stirred for 16 hours at 4°C. The solids were filtered off and washed with 50-70 ml of absolute ethanol. The filter

cake was then re-dissolved in 50 ml of distilled water, and the solution was dialyzed and finally freeze-dried to obtain 7; yield, 3.41 g, 5.7%. The supernatant was dialyzed and freeze-dried to obtain 8; yield, 0.37 g, 0.6%.

- 5 The fifth portion was ultrafiltered as described below.

Ultrafiltration:

The fifth portion from the initial extraction procedure described above (Method C) was divided into two equal parts: one was dissolved in 2 M guanidine hydrochloride (Series a), the other one in water containing 0.02% sodium azide (Series b). Both solutions were fractionated under equal conditions. The yields of the fractionations are listed in Figure 1A. Each solution was passed through a 30 KD membrane under 35 psi of nitrogen pressure until the volume of the retentate was 20 ml. Then the retentate was washed 10 times with 50 ml of 2 M GuCl (or 0.02% sodium azide); each wash was pushed through the membrane until the volume of the retentate was reduced to 20 ml and the filtrate was dialyzed against distilled water and lyophilized, yielding materials 9a,b. The thoroughly washed retentate was then passed in a similar manner through a 100 KD membrane, and the filtrate was dialyzed and lyophilized as above, yielding materials 10a,b. The resulting retentate was passed successively through a 500 KD and a 1000 KD membrane, giving 11a,b and 12a,b, respectively. Finally, the retentate was dialyzed and lyophilized to give 13a,b. The efficiency of the ultrafiltration was monitored spectrometrically to assure that the UV_{280} absorbance of final washes did not exceed 0.03.

- 30 Determination of Optimum Ammonium Sulfate Concentration:

A solution of 3 was concentrated by rotary evaporation to 50 mg/ml, split into eight equal parts, and these were treated under gentle magnetic stirring with solid ammonium sulfate. The final concentration of the precipitant ranged from 15-45% in 5% increments, and a control portion containing no ammonium sulfate was included. The mixtures were stirred for 16 hours at 4°C, then centrifuged at 20700 x g for 30 min to separate the supernatants from the pellets. The pellets and the supernatants were dialyzed separately against water and lyophilized.

Preparative Chromatography:

Sephadex™ G-100

The starting material (4, 1.0 g) was applied on a Sephadex™ G-100 column (2.6 x 100 cm) and eluted with 1 M sodium formate, pH 3.5, at a flow rate of 24 ml/h. The fractions were monitored at 280 nm and after mixing with phenol sulfuric acid reagent they were retested at 490 nm [22]. The materials were resolved into two major peaks 14 (high molecular mass peak) and 15 (low molecular mass peak).

Sephacryl™ S-300 HR

The starting material (4, 14, 1.0 g) was applied on a Sephacryl™ S-300 HR column (2.6 x 100 cm) and eluted with 1 M sodium formate, pH 3.5, at a flow rate of 24 ml/h. The fractions were monitored as above. The materials were resolved into two major peaks 16, 17 (originating from 4), and 18, 19 (originating from 14), respectively.

DEAE Sepharose™

The starting material (14, 30 mg) was applied on the column of DEAE Sepharose™ (1.0 x 20 cm) and eluted with 0.1 M sodium acetate in 10% ethanol, pH 4.4 with a linear gradient of NaCl (0.15-3.0 M), at a flow rate of 4 ml/hr.

- 5 The fractions were monitored as above. The material was resolved into three major peaks 20, 21 and 22.

Analysis:

Protein Determination

- 10 The protein determination of the samples was carried out using the BCA method [35] according to the instructions accompanying the kit in a 96-well ELISA microtitre plate format.

Amino Acid Analysis

- 15 Samples were hydrolyzed in 6M HCl under vacuum at 110°C for 20 hours, using norleucine as an internal standard. Analysis was performed on a Beckman system 6300, using the Beckman 4 x 120 mm Na-high performance hydrolysate column (cation exchange).

20 *Uronic Acid Content Determination*

Quantitative and qualitative determination of uronic acids content was performed according to Blumenkrantz and Asboe-Hansen [36]. Fresh m-hydroxydiphenyl reagent was used for each analysis.

25 *N-acetylamino sugar content*

Quantitative and qualitative determination of N-acetylamino sugar content was performed according to Reissig

etal using GalNAc and GlcNAc as standards and DMAB reagent [37].

Determination of Proteoglycan Concentration

- 5 Proteoglycan content was determined using DMB as described by Farndale et al [38]. Chondroitin sulfate A was employed as the standard.

Stimulation of splenocyte proliferation ^{脾細胞} ^{増殖}

- 10 Mouse splenocytes were isolated as previously described [39]. The cells were plated at 3×10^5 cells/well in 0.1 ml of cRPMI medium (cRPMI = complete RPMI: 90 ml RPMI 1640, 10 ml fetal calf serum, 1 ml Penicillin/Streptomycin [10,000 U/ml P, 100 µg/ml S], 1.0 ml of 0.2 M glutamine, 1.5 ml of 1 M Hepes, 0.1 ml of 2-
15 mercaptoethanol) in 96- well flat bottom tissue culture plates. Then 100 µl of each test sample in the cell medium were added to triplicate wells. ^{3倍に43} The plates were covered with sterile lids and incubated in 5% CO₂ and 100% humidity at 37°C in a CO₂ incubator for 48 hours. The cells were then
20 pulsed with ⁷⁹³H-thymidine (1 µCi /well in 10 µl cRPMI) and after incubating as above for another 18 hours they were harvested with an automated cell harvester. The filter strips were allowed to dry for 3 h at 37°C, the discs were removed from the filter strips and placed in scintillation ^{せんしゅう} ^{×花} etc.
25 vials with 7.0 ml of counting fluid and the radioactivity was then determined.

Isolation of B-cells and T-cells from splenocytes

- 30 Mouse splenocytes were isolated as above and placed in tissue culture flasks at 37°C for 2 hours, to allow the macrophages to settle and adhere to the flask. The

suspended lymphocytes were then removed, centrifuged, re-suspended in a small amount of cold PBS-EDTA-BSA buffer (PBS with 2 mM EDTA and 0.5% BSA, pH 7.4), placed on ice, counted and re-centrifuged. Finally, they were re-suspended at a concentration of 3×10^7 cells/ml in the buffer.

a) *Negative isolation of B cells*: One hundred microliters of anti-Thy-1.2-coated Miltenyi™ microbeads were added, and the mixture was incubated for 20 min. Then 5.0 ml of PBS-EDTA-BSA were added and the suspension was pipetted into a midIMACS™ column in a midIMACS™ magnet. The T-cells adhered to the column while the B cells passed through and were collected. Without removing it from the magnet, the column was rinsed with 5.0 ml of the buffer to remove any residual B-cells. The B-cells were combined, spun, re-suspended in 1 ml of cRPMI and counted. They were then diluted to 5×10^6 cells per ml of cRPMI and plated at 100 μ l/well for stimulation assays.

b) *Negative isolation of T cells*: The same procedure was followed except the magnetic beads used for the incubation were coated with anti-B220 instead of anti-Thy-1.2 antibody.

(腹膜の腹腔マクロファージ)
Nitrite Assay for mouse peritoneal macrophages

(実験動物) 子鼠の
Mice were euthanized by cervical dislocation.

Peritoneal macrophages were obtained by peritoneal lavage (洗浄) with 10 ml of ice cold cRPMI injected into the peritoneal cavity. The obtained macrophages were centrifuged for 10 min at 400 x g and re-suspended for counting. They were then re-suspended to $1-2 \times 10^6$ cells/ml of cRPMI-1640, and then plated into a 96-well tissue culture plate at 1×10^5 cells/well in a 100 μ l volume. Test samples were added in cRPMI (100 μ l) with and without IFN- γ . The positive controls were the cells plus IFN- γ (2 U/well), and LPS plus IFN- γ .

After incubating for 48 h in 5% CO₂, 50 µl of the culture fluid (LPS, 20 ng/well; IFN-γ, 2 U/well) were collected and transferred to wells in a 96-well flat bottom ELISA plate. Twofold serial dilutions of NaNO₂ (125 µM to 1 µM final concentration) in cRPMI were made, and added to a set of wells to provide a standard curve, along with 50 µl of Greiss Reagent solution each. A plot of absorbance values at 550 nm against NaNO₂ concentrations was made. The standard curve was used to determine the amount of NO₂⁻ produced by the peritoneal macrophage samples.

Stimulation of cytokine production by mouse splenocytes and determination of cytokines with ELISA

The tested substances were added at several concentrations to the cells in 96-well tissue culture plates and incubated for 24-48 hours, depending on the cytokine of interest. The supernatant culture fluid was removed and assessed by adding to 96-well ELISA plates coated with the appropriate anti-cytokine monoclonal antibody, in 50 mM carbonate buffer, pH 9.6 at 4°C overnight. The plates were then washed with TBS (2.4 g Trizma™ base, 8.0 g NaCl and 0.2 g KCl per liter, pH 7.4), post-coated with 2 mg/ml BSA in TBS (200 µl/well) for 2 hours at room temperature, and washed with TBS/Tween™ (0.05 %). One hundred µl of the unknown samples, and the standards diluted serially from 1 ng/ml to 15 pg/ml in TBS/Tween™ containing 0.1% BSA, were added to the coated ELISA plates, and incubated overnight at 4°C. After washing with TBS-Tween™, 100 µl of the appropriate biotinylated anti-cytokine mAb in PBS/Tween™, containing 0.1% BSA, was added to each well. After incubation at room temperature for 1 hour, the plates were washed with TBS/Tween™. Extravidin peroxidase in PBS/Tween™ containing 0.1% BSA was added (100 µl/well). After

incubating at room temperature for 30 min, the plates were washed and 100 μ l/well of TMB substrate solution was added. After 10 to 30 min incubation, depending on color development, the reaction was quenched with 100 μ l/well of 1
5 M H_3PO_4 , and the plate was read at 450 nm.

Effect of the extract on proliferation of Listeria monocytogenes in infected mice

Two doses of the lyophilized extract 4 prepared in accordance with Method C (0.1 mg or 4 mg) or plain water
10 were administered to Balb/c mice by intragastric tube three times a week for four weeks. The mice were then infected by intravenous injection of 5000 viable *Listeria*^{リストeria} *monocytogenes* organisms. Three days after the injections of the *Listeria* the mice were sacrificed, and cell suspensions of their
15 spleens^{脾臓} were made and cultured on culture dishes to determine the number of bacteria in the spleens of the control water fed mice and in the spleens of the mice fed with the shark cartilage extract.

Results:

20 *Extraction*

Extraction with guanidine based media or hot water yielded materials stimulating proliferation^{増殖} of splenocytes *in vitro*. Hot water extractions delivered products in higher yields. Although the products obtained from
25 guanidine based media extractions had higher proportions of proteoglycans, as seen from the uronic acid and aminosugar contents as well as from their interaction with DMB, they displayed comparable immunoactivities with those isolated from hot water extracts. The average yields of the
30 extractions and the immunoactivities of the produced extracts *in vitro* as tested on undifferentiated spleen cells,

are shown in Table 1. Table 1 provides the yield, the index of proteoglycan content, the uronic acid content and immunoactivity in vitro of shark cartilage extracts produced by Methods A, B and C. The index of proteoglycan content is defined as the ratio of the sample's proteoglycan content to the proteoglycan content of the supernatant of 40% ammonium sulfate precipitation. Uronic acid content is defined as μg glucuronic acid/ μg sample material. Immunoactivities are defined as the cell proliferation due to each sample relative to the cell proliferation due to cartilage extracted with 2 M GuCl (2). The samples were tested for immunoactivity at a concentration of 125 $\mu\text{g}/\text{ml}$.

Table 1: Comparison of three methods of extraction of immunoactive principles of shark cartilage.

method	material	yield (%)	index of proteoglycan content	uronic acid content	immunoactivity (%)
A	1	4.1	0.80	0.10	82.3
B	2	3.7	0.59	0.14	100
C	3	9.4	0.37	0.07	44.2
C	4	7.3	0.34	0.08	78.2

15

For extraction Method C, four alternatives producing extract materials 3, 4, 5, 6, 7 and 8 were compared with respect to yield and immunoactivity. Table 2 provides the results. Immunoactivities were tested at a sample concentration of 125 $\mu\text{g}/\text{ml}$ and are compared to the activity of product 2, taken as 100%.

20

Table 2: Comparison of methods for isolation of immunoactive components of hot water extract (Method C).

Method	Material	Yield (%)	Immunoactivity (%)
C i	3	9.4	44.2
C ii	4	7.3	78.2
C iii	5	4.4	92.4
C iii	6	0.5	7.0
C iv	7	5.7	80.9
C iv	8	0.6	0.1

塩析

Salting out and ethanol based fractionations

5 The active principles were precipitated from the mixtures containing more than 25% of ammonium sulfate. The immunoactivity of the pellets remained unchanged whereas the activity of the supernatants progressively decreased with increased concentration of ammonium sulfate. To obtain the highest yields of the precipitate generated from a 50 mg/ml concentration of 3 at least 35% of ammonium sulfate in the final mixture was necessary. To compensate for the variance in the starting materials we used 40% of ammonium sulfate. Although the yields of the ammonium sulfate based precipitations were compromised, the lyophilized supernatants had the highest glycosaminoglycan/proteoglycan content and were associated with the highest immunoactivities.

20 Ethanol was also examined to precipitate the immunoactive principles from concentrated hot water extracts. This method of precipitation results in higher yields and represents a simple method requiring no complicated equipment and it could be easily scaled up. The yields and changes in the specific immunoactivity in vitro are listed in Table 2.

Ultrafiltration

Much of the material dissolved in 0.02% sodium azide passed through a 30 KD membrane. Further experiments revealed that while almost all the molecules (or molecular assemblies) in the previously dialyzed extract were smaller than 1 MD, the bulk of the activity of the extract is contained in the largest molecules or molecular assemblies, i.e., those larger than 1 MD. The relevant literature dealing with SEC of PGs indicates that nonspecific adsorption is pH dependent and decreases in the presence of detergents or chaotropic agents (40). Bearing this in mind, we conducted further studies in the presence of 2 M guanidine hydrochloride. The results showed that under the conditions of exhaustive dialysis the yields of ultrafiltration did not significantly differ from those employing water preserved with sodium azide. The immunoactivity of the samples was again detected almost entirely in a region above the 100 KD range and particularly for molecules with a molecular mass greater than 1 MD (Figure 1B).

Chromatography

Size exclusion and anion exchange chromatography were executed on the crude extracts (i.e. on 4) and on selected more defined products. The eluted fractions were monitored at 280 nm for the presence of protein or protein moieties, and at 490 nm (after reaction with phenol/sulfuric reagent) for the presence of carbohydrates or carbohydrate moieties. In accordance with prior art methods, monitoring at 218 nm (absolute maximum obtained by scanning a non-chromatographed sample within 200-700 nm range) may also be done. It is close to the absorbance range used typically for monitoring proteoglycans (206-214 nm) [40]. Although only a

small local maximum at 280 was observed compared to that of BSA, it was very useful for monitoring the chromatography. A solution of bovine cartilage collagen available to us was also scanned for comparison since a significant amount of collagen was expected to be present in 4, however, due to high absorbance background at the wavelengths beyond 220 nm, no useful information was gained.

Size Exclusion Chromatography

One molar ammonium acetate, pH 6.0, resolved the crude extracts into two major peaks on Sephacryl™ S-300 HR. Surprisingly, Sephadex™ G 100, a matrix with a lower resolution range, resolved the extracts similarly into two peaks with apparently equal efficiency. Sephadex™ G-100 chromatography of dialyzed crude extracts, passed through 100 KD ultrafiltration membrane, demonstrated the competence of the ultrafiltration process. When the retained portion was chromatographed only one peak was found. When the filtrate was subjected to chromatography, a very broad peak with a small peak attached were found, confirming the presence of low molecular mass fractions contaminated only with a small portion of the high molecular mass compounds. Ammonium acetate caused problems due to its volatility under vacuum during degassing and later on was replaced with sodium acetate. Low pH also helped resolution and the concentration of the salt was found to be important; e.g. solutions lower than 0.5 M performed poorly. Final conditions for SEC utilized either 1 M sodium acetate, pH 4.4, or, preferably, 1 M sodium formate pH 3.5 (Figure 2).

Ion exchange chromatography

Our best performing system was a linear gradient of NaCl (0.15 to 3 M) in 0.1 M acetate buffer, pH 5.6.

Although this system worked with DEAE Sepharose™, there were concerns with its reproducibility. By adding 10 % EtOH in the eluent and by lowering pH to 4.4, noncovalent intermolecular interactions were significantly reduced (Figure 3). Proliferation of splenocytes challenged by the materials isolated from the chromatography was examined *in vitro*. The highest activity was found in the first peak.

Analysis

Amino acid analysis

All major amino acids were detected and the presence of D-GlcN and D-GalN was revealed in separate spiking runs. The major amino acids were glycine (15.2%), proline (14.4%), alanine (12.8%), glutamic acid (11.1%), aspartic acid (6.6%), arginine (6.2%), measured as a percentage of total amino acid content. The composition does not differ significantly among the preparations differing in molecular mass range. The only major difference was that 3, 5, and 8 contained about twice as much glycine as the dialyzed water extract 4 or the pellet obtained from EtOH precipitation (7).

Proteoglycan determination

Detection of PGs/GAGs was based on the metachromatic shift of the 590 nm peak of DMB solution to 525 nm after complexation with PGs or GAGs. This approach is a modification of a method described by Chandrasekhar [41] and facilitates rapid detection of PGs (Figure 4). Several standard curves (originally for fractions obtained from every type of process (chromatography, ultrafiltration, salt precipitation, etc) were generated. After carefully examining these curves, it was decided to use as the standard a CHS curve, defined by the equation: $y = 0.0023x$

-0.0195 ($R^2 = 0.9977$) in the concentration range 25-100 $\mu\text{g/ml}$.

Assessment of stimulation of lymphocyte proliferation

Since LPS (a powerful stimulant of lymphocyte proliferation) is a potential contaminant of biological extracts, it was essential to verify that the results were not being confounded by LPS contamination [42]. BALB/c strain, as most strains, is sensitive to LPS, so the effect of polymyxin B, a potent inhibitor of LPS on the mitogenic activity of the extracts [43,44] was assessed. In some instances the extracts were also tested on spleen cells from C3H/HeJ (insensitive to LPS strain) mice [45]. No significant differences have been found in the stimulation of BALB/c and C3H/HeJ spleen cells. Important preparations were tested at a number of concentrations in order to obtain for each sample a "titration" curve to allow a more accurate comparison of mitogenic activities between different samples (Figure 5).

Effect of the shark cartilage preparations on purified populations of B cells, T cells, macrophages, and NK cells

(マイトジェン)
(細胞分裂促進剤) Samples of the purified cells were tested for mitogen responsiveness and by flow cytometry^{血球計算}. As can be seen in Figure 6 from a representative mitogen stimulation assay of 4, the results with purified populations of cells show that the B lymphocytes are targets for induction of proliferation by the materials present in the shark cartilage preparations, whereas the T lymphocytes are not stimulated significantly. In addition it was found that macrophages were stimulated. This was of considerable interest since macrophages play a number of critical roles in the immune response.

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Purified mouse macrophages cultured with the extracts produced significantly higher levels of nitrite than macrophages challenged with a PBS blank. In Figure 7 a typical result is shown, including the evidence that the stimulation was not significantly affected by polymyxin B. Macrophages are also known to be stimulated by LPS. For example, extract 4 at two different concentrations markedly increased nitrite production by purified macrophages, and this increase was not significantly affected by the presence of polymyxin B. Conversely, although the combination of LPS and interferon γ also stimulated nitrite production, the LPS stimulation was substantially reduced in the presence of polymyxin B.

Additional experiments were carried out to further characterize the ability of the cartilage preparations to induce macrophage activation. The results confirmed that the preparations were potent inducers of macrophage activation and that this activation is greatly potentiated by co-incubation with INF- γ . Also investigated was whether the stimulation of nitrite production was due to an increase in the production per cell, or due to stimulation of proliferation of the macrophages. It was found that the extracts had no mitogenic effect on macrophages (i.e. they did not induce macrophage proliferation). The patterns of production of a number of cytokines induced by shark cartilage extract activation were examined, and an increase in production of IL-1, IL-6, IL-10, and IL-12 was demonstrated. Figure 8 shows the strong stimulatory effect of shark cartilage extracts on macrophage IL-12 and IL-6 production, again demonstrating dose-related responses.

Effect of feeding the shark cartilage extracts on susceptibility to infection with Listeria monocytogenes

Experiments were carried out on the possible protection from infection *in vivo*, using infection with *Listeria monocytogenes* as a model [46,47]. There was a significant decrease in the number of *Listeria* colony forming units in the mice fed the different extracts. As shown in Figure 9 we found that the cartilage extracts (10 mg dose) tested markedly suppressed the severity of the *Listeria* infection in terms of the number of bacterial colony forming units found in the spleen of the fed versus the non-fed animals.

Discussion:

In addition to buffered guanidine hydrochloride of various strengths, a number of other salts have been used to extract proteoglycans from cartilage. GuCl is the most popular because the others have very narrow concentration ranges for optimal extraction [32]. We did not find any statistical difference between the activity of the extracts when prepared by the above procedure (use of 2 M guanidine buffered with MOPS) or when prepared with 4 M guanidine based media, as is generally recommended in the literature [32,48]. This is an interesting finding since it is known that the size of extracted PGs is directly correlated with the concentration of the chaotropic agent [40].

Results obtained from stability studies demonstrated the exceptional thermal stability of the extracts of the present invention. Thus, when extractions were carried out at elevated temperatures or even in boiling water, it was found that the extracts could be produced in even higher yields and comparable in activity to the original, unheated guanidine extracts.

All extracts and their fractions exhibited relatively low UV absorbance at 280 nm and protein determination using the BCA method revealed less than a 25% absorbance value compared to that of the BSA standard. Low UV absorbance would suggest either effectively hindered protein cores of proteoglycans, a low frequency of aromatic residues, or a low protein content within heterogeneous preparations. Since BCA reacts with a tetradentate- Cu^+ complex (produced between the peptide bonds of the protein core of PG and Cu^{2+} in the presence of OH^-), stereochemical hindrance imposed by surrounding GAG chains could prevent the protein core of PGs from reacting with BCA. This would result in low readings and thus underestimation of the protein content. Interestingly, the protein content of about 25% was also in agreement with data obtained from amino acid analysis. Amino acid experiments indicated that both protein and amino sugars were present in the extract, supporting the presence of PGs. The composition revealing the predominance of glycine and proline suggest that all extracts, including the fractions may contain significant amounts of collagen.

Knowing which specific cell types were being stimulated was important not only for basic scientific reasons, but also because in designing potential combination therapies, combining substances which give complementary stimulation, rather than those which simply stimulate the same cells, are more likely to succeed. Activated macrophages produce nitric oxide, which is a free radical with anti-microbial activity, thus the extracts could play an important role in enhancing the body's defense against bacteria. Cytokines are known to bind to PG both to the core protein and to the GAG chain. The capacity to stimulate production of cytokines by macrophages brings important

insights with respect to the underlying mechanisms involved in the immunoenhancing activities of the shark cartilage preparations, since IL-1 has many stimulatory functions both within and outside the immune system. IL-1 promotes an acute phase response and can induce all types of cell proliferation, including tissue repair. This has broad implications, which should be considered for future studies. IL-6 is an important effector molecule involved in B lymphocyte activation and stimulation of antibody production, while IL-12 plays a major role in stimulation of NK cells and in affecting the balance of Th1 and Th2 cells [49]. This is important because Th1 cells are primarily involved in cellular immunity and Th2 cells appear primarily involved in humoral immunity.

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CLAIMS:

1. A composition from shark cartilage comprising proteoglycan and an immunoactive component having a molecular weight greater than about 100 KD, wherein the composition has a uronic acid content of about 0.05-0.5 µg glucuronic acid per µg of the composition.
70=酸, suger 骨格
2. The composition according to claim 1, wherein the immunoactive component has an average molecular weight of about 500 KD.
3. The composition according to claim 1, wherein the immunoactive component has a molecular weight greater than about 1 MD.
4. The composition according to any one of claims 1 to 3, wherein the uronic acid content is about 0.08-0.3 glucuronic acid per µg of the composition.
5. The composition according to any one of claims 1 to 4, further comprising xylose, rhamnose, galactose, glucose or mixtures thereof.
6. The composition according to any one of claims 1 to 5, further comprising aminosugars.
7. The composition according to any one of claims 1 to 6, further comprising about 20-30% protein by weight of the composition.
8. The composition according to any one of claims 1 to 7, wherein glycine content is about 14-16% by weight of total amino acids, proline content is about 14-15% by weight of total amino acids, alanine content is about 12-14% by weight of total amino acids, glutamic acid content is about 10-12% by weight of total amino acids, aspartic acid content

is about 6-7% by weight of total amino acids and arginine content is about 5-7% by weight of total amino acids.

9. The composition according to any one of claims 1 to 8, wherein the immunoactive component comprises
5 proteoglycan.

10. A formulation comprising an immunomodulating effective amount of a composition according to any one of claims 1 to 9 together with a physiologically acceptable carrier, excipient and/or diluent.

10 11. The formulation according to claim 10 adapted for oral administration.

12. The formulation according to claim 10 or 11 for treating or preventing tumor growth, bacterial infections, viral infections and/or fungal infections.

15 13. Use of an immunomodulating effective amount of a composition according to any one of claims 1 to 9 for modulating an immune system of a subject in need of immunomodulation.

(20 14. Use of an immunomodulating effective amount of a composition according to any one of claims 1 to 9 for preparing a medicament for modulating an immune system of a subject in need of immunomodulation.

15. The use according to claim 13 or 14, wherein the composition is used orally.

25 16. The use according to any one of claims 13 to 15 for treating or preventing tumor growth, bacterial infections, viral infections and/or fungal infections.

17. A commercial package comprising an immunomodulating effective amount of a composition according

to any one of claims 1 to 9 together with instructions for its use in modulating an immune system of a subject in need of immunomodulation.

18. The commercial package according to claim 17,
5 wherein the composition is adapted to be used orally.

19. The commercial package according to claim 17 or 18, wherein the instructions are for use of the composition for treating or preventing tumor growth, bacterial infections, viral infections and/or fungal infections.

10 20. A method comprising administering to a subject in need of immunomodulation an immunomodulating effective amount of a composition according to any one of claims 1 to 9.

15 21. The method according to claim 20, wherein the composition is administered orally.

22. The method according to claim 20 or 21 the immunomodulation is for treating or preventing tumor growth, bacterial infections, viral infections and/or fungal infections.

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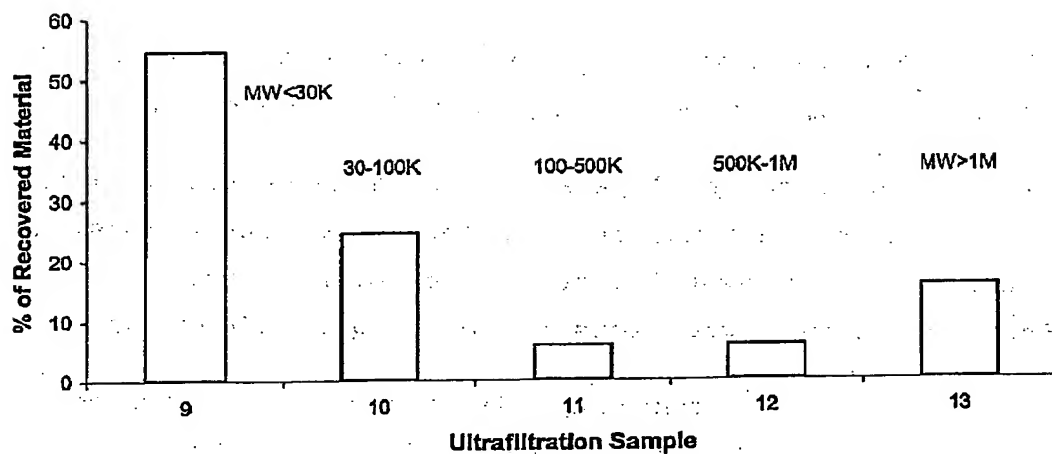


FIG. 1A

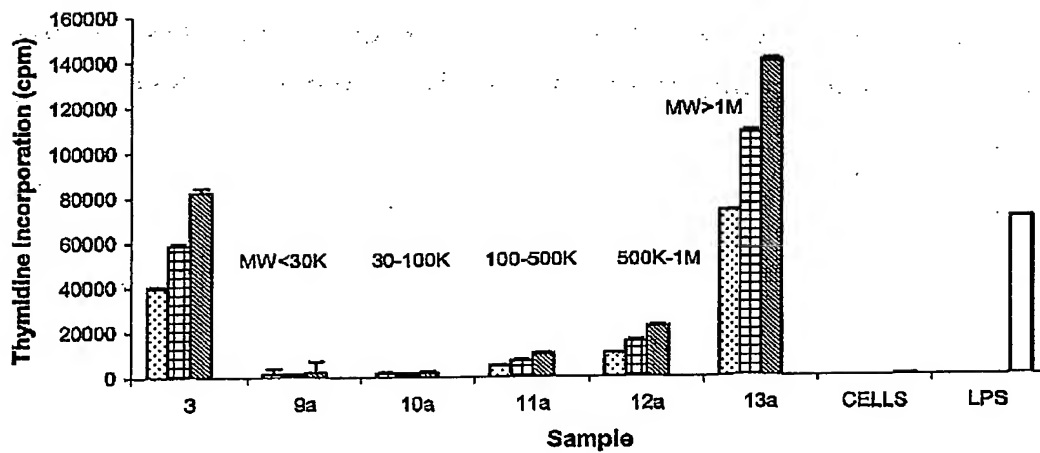


FIG. 1B

本願では 500-1000D
のものが most active
なため、組成が異なるものは

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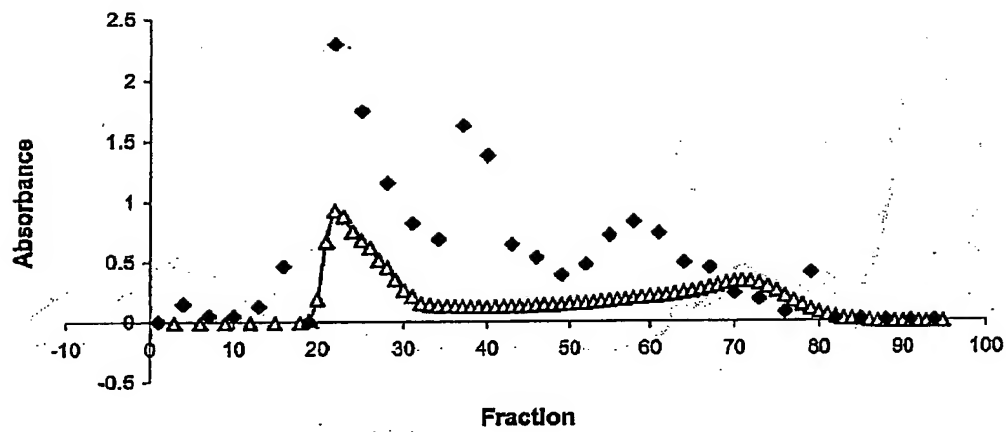


FIG. 2

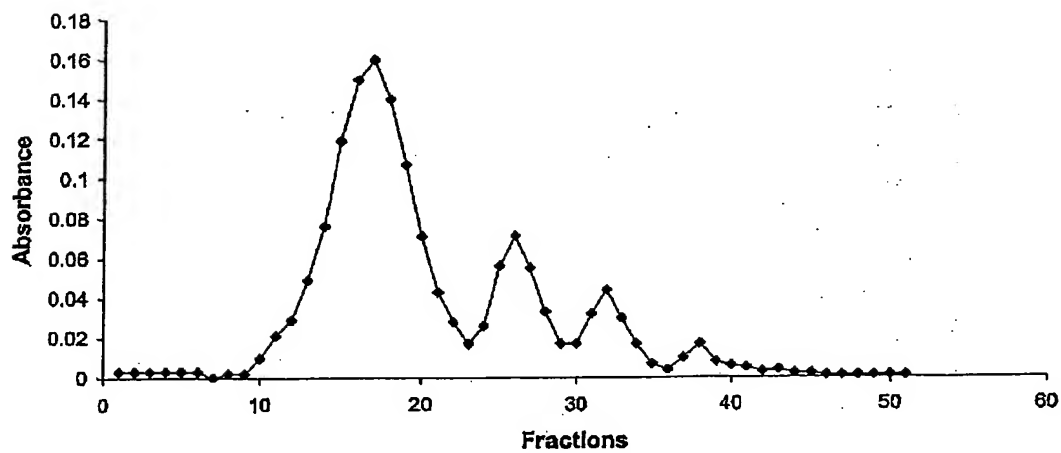


FIG. 3

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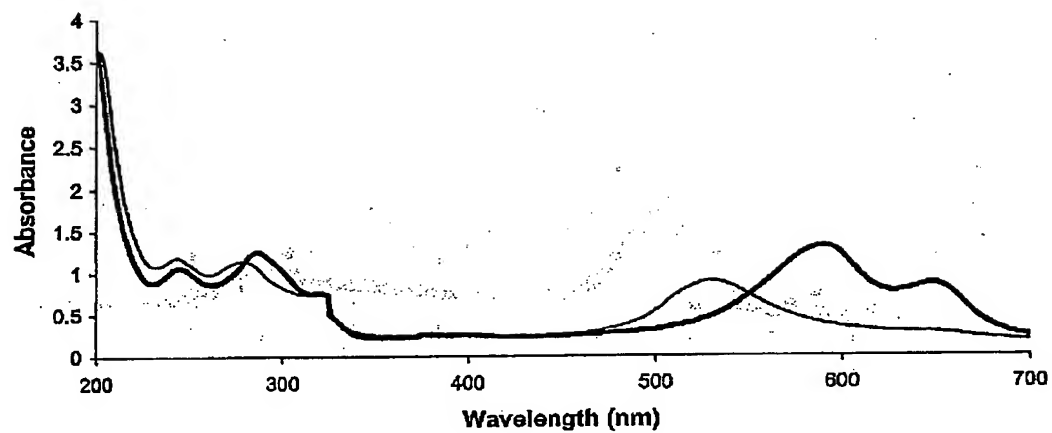


FIG. 4

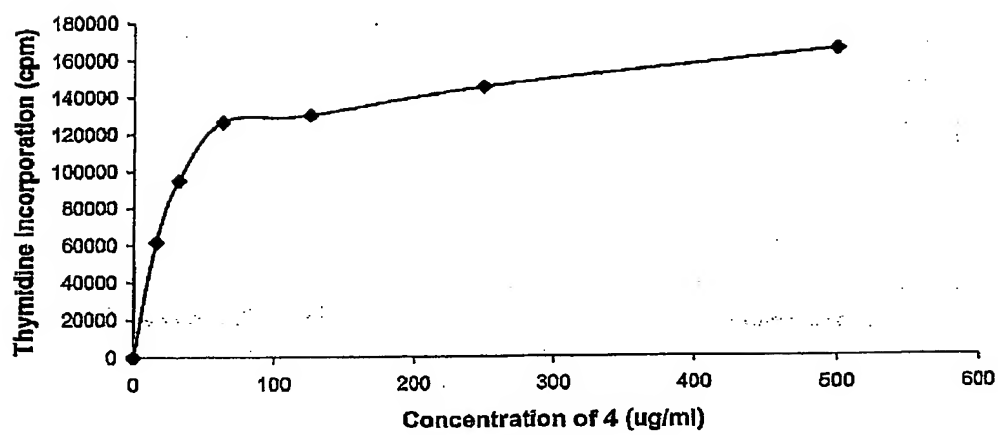
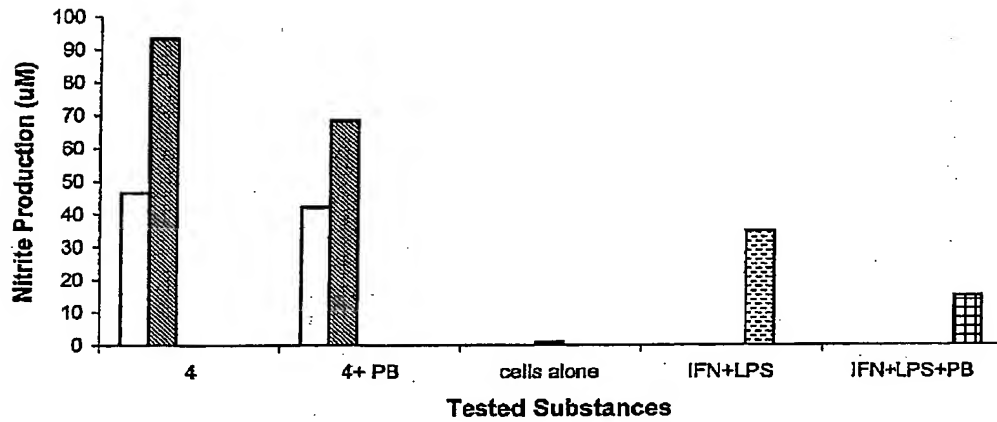
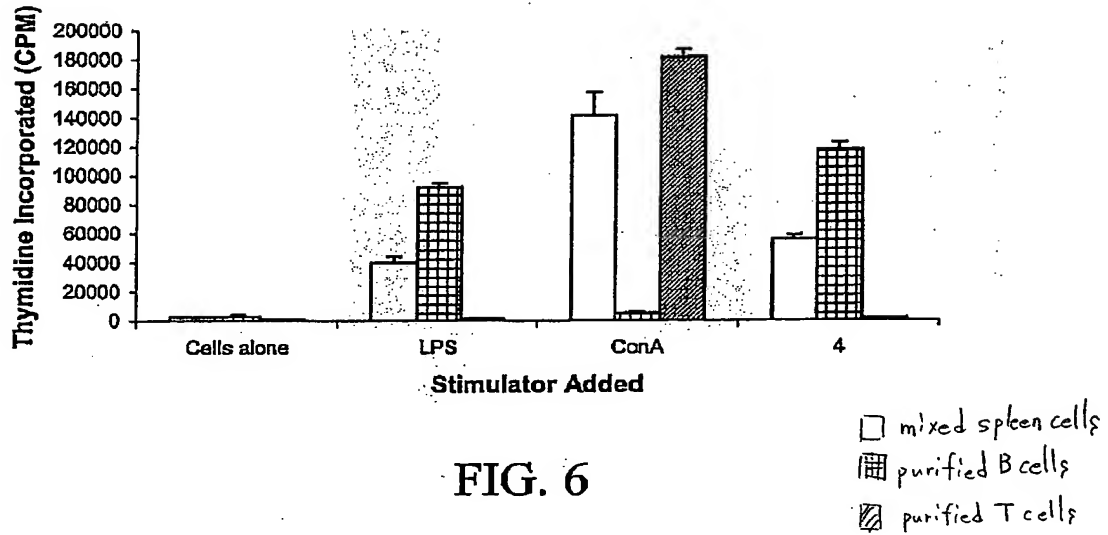


FIG. 5

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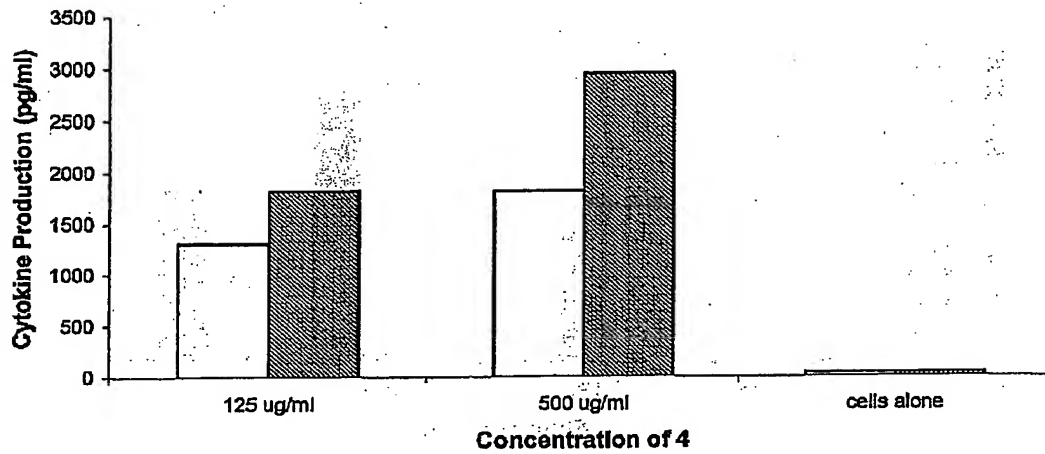


FIG. 8

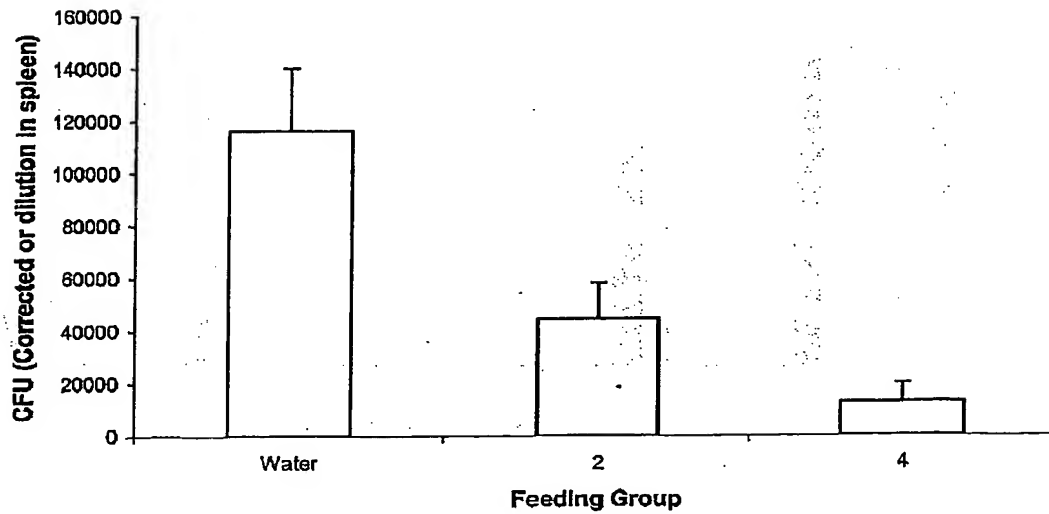


FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 02/00174

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 A61K35/60 A61K35/32 A61P37/02 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data, MEDLINE, EMBASE, BIOSIS, PASCAL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 16197 A (AETERNA LAB INC) 9 May 1997 (1997-05-09) page 9, line 20 - line 25; claims 18,29,42 page 50 page 51, line 8	1-11, 17-21
X	CA 2 201 025 A (BRUNDRITT LARRY WAYNE) 26 September 1998 (1998-09-26) abstract page 1, line 23 - page 2, line 4 page 2, line 12 - line 21; claim 1	1,10-22
X	US 4 473 551 A (SCHINITSKY MICHAEL) 25 September 1984 (1984-09-25) cited in the application abstract; example 1	1,10-22
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) -
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

31 October 2002

Date of mailing of the international search report

15/11/2002

Name and mailing address of the ISA

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 Fax (+31-70) 340-3016

Authorized officer

Laffargue-Haak, T

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 02/00174

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 2001 GIESE S ET AL: "Influence of the oral ingestion of shark cartilage and rice bran on the immune system in a rat model." Database accession no. PREV200100483327 XP002219069 abstract & SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 27, no. 1, 2001, page 22 31st Annual Meeting of the Society for Neuroscience; San Diego, California, USA; November 10-15, 2001 ISSN: 0190-5295</p>	1,10-22
X	<p>SIVAKUMAR P ET AL: "Occurrence of a novel collagen with three distinct chains in the cranial cartilage of the squid Sepia officinalis: comparison with shark cartilage collagen" BBA - GENERAL SUBJECTS, ELSEVIER SCIENCE PUBLISHERS, NL, vol. 1381, no. 2, 23 July 1998 (1998-07-23), pages 161-169, XP004276159 ISSN: 0304-4165 figure 2</p>	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 02/00174

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 13, 20-24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 02/00174

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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US 4473551	A	25-09-1984	NONE	